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Moreover, Shi et al. does not disclose such aptamers. It discloses an expression system for producing a multivalent aptamer, i.e., a plurality of monomer aptamers linked together to produce a product that has the ability to bind 5 units of its target protein. In the process described in Shi et al., the transcribed RNA consists of five monomeric units of aptamers that are connected tandemly. Because of this, the transcribed RNA has the potential to bind to a target at 1:5 RNA to target ratio. In the multivalent aptamer disclosed by Shi et al., each individual monovalent aptamer component of the multivalent aptamer has the ability to bind to a target sequence.

In contrast, the present claims relate neither to expression of aptamers nor to producing an active full-length aptamer composed of active subunit aptamers. The present invention involves two inactive aptamer oligonucleotides, i.e., two unconnected oligonucleotides (derived from one active aptamer) neither of which on its own has the ability to bind to the target molecule. The two oligonucleotides undergo a conformational changes only in the presence of the target molecule. This is the reason why they are referred to as modulating (or modulate) aptamers. These modulating aptamers have advantages over full-length aptamers as diagnostic agents. For example, use of full-length aptamers in diagnosis requires special labeling or modifications in order to discriminate bound from unbound aptamer. On the other hand discrimination of target-bound versus unbound modulate aptamers is facilitated by the fact that fluorescence is only emitted following binding of the two modulate aptamers to the test target molecule. Applicants were the first to observe that two inactive oligonuclotides derived from an active aptamer could be reconstituted in the presence of the target molecule. The novelty of Applicant's discovery is documented in a recently published article (Nutiu et al., J. Am. Chem. Soc. 125:4771-4778, 2003). The first two pages of this article are enclosed as Exhibit A and the relevant text on page 4772 is circled. If the Examiner would like a copy of the whole article, Applicants would be pleased to supply one.

In light of the above considerations, Applicants respectfully submit that the claims of Groups 1-3 do relate to a single general inventive concept under PCT Rule 13.1 and thus request that all the pending claims be examined in the present application.

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Respectfully submitted,

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Structure-Switching Signaling Aptamers

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Abstract: Aptamers are single-stranded nucleic acids with defined tertiary structures for selective binding to target molecules. Aptamers are also able to bind a complementary DNA sequence to form a duplex structure. In this report, we describe a strategy for designing aptamer-based fluorescent reporters that function by switching structures from DNA/DNA duplex to DNA/target complex. The duplex is formed between a fluorophore-labeled DNA aptamer and a small oligonucleotide modified with a quenching moiety (denoted QDNA). When the target is absent, the aptamer binds to QDNA, bringing the fluorophore and the quencher into close proximity for maximum fluorescence quenching. When the target is introduced, the aptamer prefers to form the aptamer-target complex. The switch of the binding partners for the aptamer occurs in conjunction with the generation of a strong fluorescence signal owing to the dissociation of QDNA. Herein, we report on the preparation of several structure-switching reporters from two existing DNA aptamers. Our design strategy is easy to generalize for any aptamer without prior knowledge of its secondary or tertiary structure, and should be suited for the development of aptamer-based reporters for real-time sensing applications.

Introduction

Aptamers are single-stranded nucleic acids isolated from random-sequence nucleic acid libraries by "in vitro selection".[1,2] To date, numerous aptamers have been created for a broad range of targets, including metal ions, small organic compounds, metabolites, and proteins.[3,4] The tight-binding capabilities of both DNA and RNA aptamers have been demonstrated in numerous cases including a 2'-aminopyrimidine-containing RNA aptamer for vascular permeability factor/vascular endothelial growth factor with a K_d of 0.14 nM,[5] a 2'-fluoro-modified RNA aptamer for the human keratinocyte growth factor with K_d of 0.3 pM,[6] and a DNA aptamer for platelet-derived growth factor-AB with subnanomolar affinity.[7] Aptamers can also be made to possess a high binding specificity, exemplified by an anti-theophyllin RNA aptamer[8] that displays a >10 000-fold discrimination against caffeine (which differs theophyllin by a methyl group) and an anti-L-arginine RNA aptamer that exhibits a 12 000-fold affinity reduction toward D-arginine.[9] The target versatility, the high binding affinity and specificity, along with the simplicity of in vitro selection, make aptamers attractive as molecular tools for bioanalytical applications. In such cases, it is advantageous if aptamers are able to report on target presence by real-time fluorescence signaling without a need for complex separation steps.

Standard DNA and RNA molecules do not contain intrinsically fluorescent groups. To make aptamers fluoresce, it is necessary to modify aptamers with extrinsic fluorophores. Considerable research activities aiming at designing real-time signaling aptamers have been reported recently. One strategy is to covalently attach a fluorophore at a location of an aptamer that will undergo a target-induced conformational change.[10,11] Such reporters can be created either by rational design if tertiary structure information is available[10] or by in vitro selection using a fluorophore-labeled library.[11] A critical assumption in this approach is that the conformational change might substantially alter the electronic environment of the attached fluorophore to cause a significant change in its fluorescence property. Because of the difficulty in precisely predicting (1) whether the attachment site will undergo a significant conformational change upon target binding and (2) whether such a change could indeed alter the fluorescence property of the attached fluorophore, many

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rationally designed constructs[10] or selected aptamers[11] may have to be tested before a desirable signaling aptamer can be obtained. Therefore, this strategy is not easy to generalize. Furthermore, the known signaling aptamers made by this approach usually exhibit fairly small fluorescence enhancements upon target binding (typically below 2-fold at saturating target concentrations[10,11]) and consequently their detection sensitivity is relatively low.

Other studies have focused on designing molecular beacon-based signaling aptamers (denoted "aptamer beacons")[12-16] through the adaptation of the molecular beacon concept originally designed for the detection of nucleic acid targets by nucleic acid hybridization.[17] Yamamoto et al. reported the first aptamer beacon designed from an RNA aptamer that interacts with the Tat protein of HIV-1.[12] These researchers split the aptamer into two molecules, one of which was formulated into a hairpin-shaped beacon molecule (after the addition of a few nucleotides to tie the two ends of the RNA into a hairpin structure and the attachment of a fluorophore at one end of the RNA and a quencher at the other end). In the absence of Tat, the two RNA molecules exist independently; the beacon half of the aptamer adopts the hairpin structure, emitting a low level of fluorescence. When Tat is introduced, the beacon changes its structure in order to engage the other half of the aptamer for binding to Tat; the disruption of the hairpin structure causes physical separation of the fluorophore–quencher pair, resulting in a fluorescence enhancement. The successful design of the above signaling aptamer is achieved because the original RNA aptamer has a unique secondary structure that contains a long stretch of paired nucleotides to permit the splitting of the aptamer into two molecules. Therefore, it can be difficult to use the same strategy for other aptamers that lack such a secondary structure feature. An alternative molecular beacon strategy has been reported by Hamaguchi et al.[13] in which they place an intact aptamer as the loop segment of a molecular beacon. However, this strategy is difficult to generalize as well, particularly for large aptamers and the aptamers in which the two ends of the aptamer sequence do not move away from each other after target binding (e.g., the anti-ATP DNA aptamer[14,15]). Moreover, tying the two ends of an aptamer into a hairpin structure could significantly alter the correct tertiary folding of the aptamer and consequently, such a modified aptamer may lose its binding ability. For example, only one of the three anti-thrombin aptamer beacons designed by Hamaguchi et al.[13] based on a known anti-thrombin DNA aptamer[20] was able to retain the thrombin-binding ability, whereas the other two failed to interact with thrombin completely.[13] Considering that aptamers have variable sizes and different kinds of secondary structures and that many aptamers may not have an easily determined secondary structure, there is an obvious need to establish a signaling-aptamer designing strategy that is easy to generalize and has little restrictions on the size and secondary structure of aptamers.

Herein, we describe a simple and general approach for preparing solution-based signaling aptamers that function by a coupled structure-switching/fluorescence-dequenching mechanism. Our strategy exploits the unique ability of each DNA aptamer to adopt two distinct structures—a DNA duplex with a complementary DNA sequence, and a tertiary complex with the target for which the aptamer is created. Our signaling aptamers take advantage of target-induced switching between a DNA/DNA duplex and a DNA/target complex. Generation of a signal upon formation of the DNA/target complex is obtained by using a fluorophore-labeled DNA aptamer and a small complementary oligonucleotide that is covalently modified with a quencher (denoted QDNA). In the absence of the target, the aptamer naturally binds to the QDNA, bringing the fluorophore and the quencher into close proximity for highly efficient fluorescence quenching. When the target is introduced, the aptamer prefers to form the aptamer-target complex rather than the aptamer-QDNA duplex, triggering the release of the QDNA from the fluorophore-labeled aptamer. The dissociation of the QDNA is accompanied by the increase of fluorescence intensity because of fluorescence dequenching. On the basis of this strategy, we have successfully engineered several fluorescent reporters from two existing DNA aptamers, one that is specific for ATP and the other that binds thrombin.

Experimental Section

DNA Oligonucleotides and Chemical Reagents. Standard and modified DNA oligonucleotides were all prepared by automated DNA synthesis using cyanoethylphosphoramidite chemistry (Keck Biotechnology Resource Laboratory, Yale University; Central Facility, McMaster University). 5′-Fluorescein and 3′-DABCYL (4-(4-dimethylaminophenylazo)benzoic acid) moieties were introduced using 5′-fluorescein phosphoramidite and 3′-DABCYL-derivatized controlled pore glass (CPG) (Glen Research, Sterling, Virginia) and were purified by reverse phase HPLC. HPLC separation was performed on a Beckman-Coulter HPLC System Gold with a 168 Diode Array detector. The HPLC column was an Agilent Zorbax ODS C18 Column, with dimensions of 4.5 × 250 mm and a 5 μm bead diameter. A two-solvent system was used for the purification of all DNA species, with solvent A being 0.1 M triethylammonium acetate (TEAA, pH 6.5) and solvent B being 100% acetonitrile. The best separation results were achieved by a nonlinear elution gradient (10% B for 10 min, 10%B to 40%B over 45 min) at a flow rate of 0.5 mL/min. The main peak was found to have very strong absorption at both 260 and 491 nm. The DNA within 2/3 of the peak width was collected and dried under vacuum. Unmodified DNA oligonucleotides were purified by 10% preparative, denaturing (8 M urea) polyacrylamide gel electrophoresis (PAGE), followed by elution and ethanol precipitation. Purified oligonucleotides were dissolved in water and their concentrations were determined spectroscopically. Human factor Xa and human factor IIa were purchased from Haematologic Technologies (Essex Jct., VT). Human thrombin, bovine serum albumin (BSA), adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), deoxyadenosine 5′-triphosphate (dATP), uridine 5′-triphosphate (UTP), cytosine 5′-triphosphate (CTP), guanosine 5′-triphosphate (GTP), cytosine 5′-triphosphate (CTP),

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